Stability Indicating HPTLC Method for Estimation of Dabigatran Etexilate Mesylate in its Pharmaceutical Dosage Form

Pintu B. Prajapati  
Maliba Pharmacy College, INDIA  

Arti J. Rakholiya  
Maliba Pharmacy College, INDIA  

Kunj B. Bodiwala  
Maliba Pharmacy College, INDIA  

Bhavin P. Marolia  
Maliba Pharmacy College, INDIA  

Shailesh A. Shah  
Maliba Pharmacy College, INDIA  

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ABSTRACT
Dabigatran etexilate mesylate is an anticoagulant drug. Dabigatran demonstrated its efficacy for prophylaxis and treatment of thromboembolic event during orthopaedic surgery and curative treatment of hypercoagulability in atrial fibrillation. The present study deals with the development and validation of a stability-indicating high performance thin-layer chromatography (HPTLC) method for the estimation of Dabigatran etexilate mesylate using TLC plates precoated with silica gel 60 F254 as stationary phase and toluene: ethyl acetate: methanol: formic acid (3:4:3:0.2, v/v/v/v) as the mobile phase. The drug was subjected to stress conditions such as hydrolysis, oxidation, photolysis, neutral and dry heat. Degradation products produced as a result of the stress conditions did not interfere with the detection of DEM, therefore the proposed method can be considered stability-indicating. DEM showed degradation under hydrolytic, oxidative, photolytic and dry heat conditions. DEM (Rf 0.47 ± 0.02) and its degradation products were well resolved. The wavelength selected for quantitation was 314 nm. The method was linear in the concentration range 50–250 ng/spot with a correlation coefficient of 0.9955. The %RSD for repeatability of peak area measurement was found to be 0.62 and %RSD for repeatability of sample application was found to be 0.75. The % RSD of intraday and interday precisions were 0.91- 1.5 and 1.21- 1.7 respectively. The accuracy (recovery) was found to be in the range of 99.45–100.37 %. The developed method was applied for assay of marketed formulations and the results were found to be good agreement with labelled claim of formulations.

Keywords: high-performance thin-layer chromatography (HPTLC), dabigatran etexilate mesylate (DEM), stress degradation, method validation
INTRODUCTION

Dabigatran etexilate mesylate is an anticoagulant drug. DEM (Figure 1) is chemically β-Alanine, N-[[2-[[4-[[((hexyloxy) carbonyl] amino] imino methyl] phenyl] amino]-1-methyl-1H benzimidazol 5-yl] carbonyl]-N-2 pyridinyl, ethyl ester, methane sulfonate. Dabigatran demonstrated its efficacy for prophylaxis and treatment of thromboembolic event during orthopedic surgery and curative treatment of hypercoagulability in atrial fibrillation [1, 2].

![Chemical structure of DEM](image)

Figure 1. Chemical structure of DEM

Stability-indicating methods (SIM) are among the essential tools included in the international council on harmonization (ICH) guidelines. Establishment of a stability indicating method is mandatory for study of degradation pathways and intrinsic stability, and separation of a drug from its degradation products is part of validation of an analytical method. Stability-indicating methods are performed by means of chromatographic techniques, for example HPLC and HPTLC. Forced degradation studies are conducted under a variety of conditions, and include hydrolysis, thermal, photolytic, and oxidative degradation [3].

HPTLC methods are cost-effective because, unlike HPLC, several samples can be run simultaneously using a small quantity of mobile phase, thus reducing analysis time and cost per analysis. Mobile phase of pH 8 and above can be used. Suspensions and dirty or turbid samples can be applied directly to a plate. This facilitates automated application and scanning in situ. HPTLC facilitates repeated detection (scanning) of the chromatogram under the same or different conditions. Simultaneous assay of several components in a multicomponent formulation is possible [4].

There are few analytical methods reported in literatures for analysis of DEM which includes UV and chromatography [5]. Literature also describes the estimation of DEM in presence of its degradation products by HPLC method [6-10], UPLC MS/MS in human plasma [11], LC/MS based metabolite identification and semi quantitative estimation approach in the
investigation of in vitro dabigatran etexilate metabolism [12-16]. But no literature described stability-indicating HPTLC method for analysis of DEM as active pharmaceutical ingredient or in dosage forms. Hence, the main objective of this work was to develop and validate stability-indicating HPTLC method for estimation of DEM in its pharmaceutical dosage forms.

EXPERIMENTAL

Chemicals and Reagents

DEM was obtained as a gift sample by Glenmark Pharmaceuticals Ltd. (Bharuch, Gujarat). Toluene, Methanol, Ethyl acetate, Formic acid, NaOH pellets, HCl, Hydrogen Peroxide, 6 % v/v (All reagents were purchased from SD Fine Chemicals Limited, Mumbai, India) were used. Commercial capsule formulations of DEM were (PRADAXA 110 mg capsule manufactured by Boehringer Ingelheim Pharmaceuticals, Germany) procured from market.

Preparation of solutions

Preparation of working standard solution of DEM

Stock solution of DEM (1000 µg/ml) was prepared by dissolving 10 mg of drug in 10 ml of methanol. From the standard stock solution, 1 ml was transferred into 10 ml volumetric flask and diluted up to the mark with methanol (100µg/ml). From the resulting solution, 1 ml was transferred into 10 ml volumetric flask and diluted up to the mark with methanol to obtain a solution having strength of 10µg/ml.

Preparation of forced degradation sample

Acidic Hydrolysis: Accurately weighed 10 mg of DEM was dissolved and diluted up to the mark with 1N HCl in 10 ml volumetric flask. The solution was kept for half an hour at 27±2°C. Aliquot of 1 ml was transferred to 10 ml volumetric flask, neutralized with 1N NaOH and diluted up to mark with methanol. From the above solution 1 ml was transferred to 10 ml volumetric flask and volume was made up to 10 ml with methanol.

Alkaline Hydrolysis: Accurately weighed 10 mg of DEM was dissolved and diluted up to the mark with 0.01N NaOH in 10 ml volumetric flask. The solution was kept for half an hour at 27±2°C. Aliquot of 1 ml was transferred to 10 ml volumetric flask, neutralized with 0.01N HCl and diluted up to mark with methanol. From the above solution 1 ml was transferred to 10 ml volumetric flask and volume was made up to 10 ml with methanol.

Oxidative Degradation: Accurately weighed 10 mg of DEM dissolved and diluted up to the mark with 1% hydrogen peroxide in 10 ml volumetric flask. The solution was kept for 1hr. at 27±2°C. Aliquot of 1 ml was transferred to 10 ml volumetric flask and volume was made up to mark with methanol. From the above solution 1 ml was transferred to 10 ml volumetric flask and volume was made up to 10 ml with methanol.
Neutral Condition: Accurately weighed 10 mg of DEM was transferred, dissolved and diluted up to the mark with distilled water in 10 ml volumetric flask. The solution was heated for 8 hrs at 80°C and cooled. Aliquot of 1 ml was transferred to 10 ml volumetric flask and volume was made up to mark with methanol. From the above solution, 1 ml was transferred to 10 ml volumetric flask and volume was made up to the mark with methanol.

Photolytic Degradation: Accurately weighed 10 mg of DEM was exposed to direct sunlight for 24 hrs. Then it was transferred, dissolved and diluted up to the mark with methanol in 10 ml volumetric flask. Aliquot of 1 ml was transferred to 10 ml volumetric flask and volume was made up to mark with methanol. From the above solution, 1 ml was transferred to 10 ml volumetric flask and volume was made up to 10 ml with methanol.

Dry Heat Degradation: Accurately weighed 10 mg of DEM was taken in porcelain dish and kept in the hot air oven at 110°C for 8 hrs. After that, it was transferred into 10 ml volumetric flask, dissolved and diluted up to the mark with methanol. Aliquot of 1 ml was transferred to 10 ml volumetric flask and volume was made up to mark with methanol. From the above solution, 1 ml was transferred to 10 ml volumetric flask and volume was made up to the mark with methanol.

Chromatographic condition

Chromatographic separation was performed on 10 cm × 10 cm aluminium backed TLC plates coated with 250 µm layers of silica gel 60F254 (E. Merck, Germany). Samples were applied as 6 mm bands, 10 mm apart, by means of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a Hamilton syringe. Samples were applied from the left edge of the plate at a constant rate of application of 0.1 μl/sec. Linear ascending development to a distance of approximately 75 mm was performed in a Camag 10 cm × 10 cm twin trough glass chamber using toluene: ethyl acetate: methanol: formic acid (3:4:3:0.2, v/v/v/v) as mobile phase. Before development, the chamber was saturated with the vapor of mobile phase. The optimum saturation time was 30 min. After development the plates were dried then scanned at 314 nm with a Camag TLC scanner III in absorbance- reflectance mode. The slit dimensions were 4.00 x 0.30 mm, the scanning speed 20 mm/sec. and the source of radiation was a deuterium lamp.

Solution Stability

Freshly prepared working solution of DEM (10μg/ml) was stored at 27±2°C. The standard solution was analyzed after 24 hours and compared with the initial peak area.

Procedure for calibration curve

From working standard solution of DEM, aliquots of 5, 10, 15, 20 and 25 μl were spotted on the TLC plate and analysed as described under section 2.3. The calibration curves of peak area versus respective concentration were plotted and correlation coefficient and regression line equation was computed.
Method Validation

The method was validated as per ICH guidelines [17].

Linearity

Different concentrations of DEM (50-250 ng/spot) were applied on five different TLC plates, developed, dried and the peak areas were measured as described in chromatographic condition. The calibration curve was constructed by plotting graph of the mean peak area versus respective concentration of DEM and correlation coefficient and the regression line equation was derived.

Precision

Repeatability of peak area measurement: From working standard solution of DEM, 15 µl was spotted on TLC plate, developed, dried and analysed as described under chromatographic condition. The obtained band was scanned for seven times and % CV for peak area measurement was calculated.

Repeatability of sample application: From working standard solution of DEM, 15 µl was spotted seven times on TLC plate, developed, dried and analysed as described under chromatographic condition. The % CV for sample application was calculated.

Intra-Day and Inter-Day Precision: From working standard solution of DEM, 10, 15 and 20 µl were spotted on pre-coated TLC plate under nitrogen stream using Linomat V semi-automatic sample applicator. The spotted plate was developed, dried and analysed as described in chromatographic condition. Intra-day precision of the proposed method was evaluated by repeating procedure three times on same day and % CV was calculated. Inter-day precision of the proposed method was evaluated by repeating procedure on three consecutive days and % CV was calculated.

Accuracy

Accuracy was determined in terms of percentage recovery. The proposed method applied to determine DEM in its pharmaceutical dosage form. The recovery experiment was carried out in triplicate by spiking previously analysed samples of DEM with three different concentrations of respective standards at 80 %, 100 % and 120 %.

Limit of Detection and Limit of Quantitation

LOD and LOQ of the drug were calculated using following equations as per ICH guideline

\[
LOD = 3.3 \times \sigma / S
\]

\[
LOQ = 10 \times \sigma / S
\]
where, $\sigma$ is the standard deviation of y-intercept, $S$ is the mean slope of the five calibration curves.

Specificity

To confirm the specificity of proposed method, from both working standard solution of DEM and sample solution of DEM tablet, 15 μl were spotted on same TLC plate. The spotted plate was developed, dried and scanned as described in chromatographic condition. The spots of both standard drugs from tablet were confirmed by comparing its $R_f$ and absorbance-reflectance UV spectrum with that of respective standard DEM. The chromatographic peak purity of drug was confirmed by correlating the UV spectra of standard DEM and DEM from the dosage form scanned at peak start, peak apex, and peak end positions of the spots.

Analysis of forced degraded sample

From each forced degraded sample solution of DEM, aliquots of 25 μl was spotted on the TLC plate and analysed as described under section Chromatographic condition.

Analysis of DEM in Marketed Formulation

Twenty capsules were emptied, weighed, triturated and mixed. Accurately weighed powder equivalent to 10 mg of DEM was transferred to 10 ml volumetric flask, 5 ml methanol was added, sonicated for 10 min, diluted with methanol up to mark, mixed well and filtered through Whatman filter paper no. 41. From the above solution, 1 ml was transferred to 10 ml volumetric flask and volume was made up to the mark with methanol. From the resulting solution, 1 ml was transferred to 10 ml volumetric flask and volume was made up to the mark with methanol. A volume of 15 μl of above solution was applied on a TLC plate, developed and analysed. Concentration of drug was calculated using straight line equation of calibration curve. The solution was analysed in triplicate.

Figure 2. Chromatogram showing the standard DEM (250 ng/spot)
RESULTS AND DISCUSSION

Optimization of mobile phase

For the optimization of mobile phase, different solvent systems have been tried alone and in combination (methanol, toluene, ethyl acetate, dichloromethane etc.). DEM standard solutions and forced degraded sample in different conditions were spotted on the TLC plates and run in different solvent systems. The mobile phase toluene: ethyl acetate: methanol: formic acid (3:4:3:0.2, v/v/v/v) showed good resolution and compact spots with $R_f$ value of 0.47 for DEM. This mobile phase was able to separate all the degradation products of DEM obtained at different stress conditions. The chromatogram for standard DEM is shown in Figure 2.

Solution Stability

Solution was kept at room temperature (27±2°C) for 24 hours and analyzed by HPTLC method of DEM. No significant change was observed in peak area of chromatogram, implies that solution of drug was stable up to 24 hours at room temperature.

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**Table 1.** Calibration data for linearity

<table>
<thead>
<tr>
<th>Conc. (ng/spot)</th>
<th>Area (Mean ± SD) (n=5)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1585.8 ± 18.18</td>
<td>1.14</td>
</tr>
<tr>
<td>100</td>
<td>3093.5 ± 39.70</td>
<td>1.28</td>
</tr>
<tr>
<td>150</td>
<td>4292.0 ± 50.87</td>
<td>1.18</td>
</tr>
<tr>
<td>200</td>
<td>5460.8 ± 27.27</td>
<td>0.49</td>
</tr>
<tr>
<td>250</td>
<td>6482.8 ± 20.63</td>
<td>0.32</td>
</tr>
</tbody>
</table>

**Table 2.** Precision of the analytical method

<table>
<thead>
<tr>
<th>Concentration of DEM (ng/spot)</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak area Mean ± SD (n=3)</td>
<td>%RSD</td>
</tr>
<tr>
<td>100</td>
<td>3074.33 ± 46.32</td>
<td>1.50</td>
</tr>
<tr>
<td>150</td>
<td>4325.70 ± 62.68</td>
<td>1.44</td>
</tr>
<tr>
<td>200</td>
<td>5475.00 ± 50.08</td>
<td>0.91</td>
</tr>
</tbody>
</table>

**Table 3.** Recovery study of the analytical method

<table>
<thead>
<tr>
<th>DEM from pre analysed capsule powder (mg)</th>
<th>Std. DEM spiked (mg)</th>
<th>Conc. of DEM in final Solution (µg/ml)</th>
<th>Recovered amount of DEM (mg)</th>
<th>% Recovery ± S.D. (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>10</td>
<td>9.95</td>
<td>99.5±1.01</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>18</td>
<td>8.02</td>
<td>100.37±1.44</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>20</td>
<td>10.03</td>
<td>100.37±1.52</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>22</td>
<td>11.93</td>
<td>99.45±0.87</td>
</tr>
</tbody>
</table>
Method Validation

The response for the drug was found to be linear in the concentration range 50-250 ng/spot for DEM with correlation coefficient of 0.9955 (Table 1). The %RSD values were 0.91-1.5 % and 1.21-1.7 % for intraday and interday precision, respectively (Table 2) and % RSD for repeatability of peak area measurement was found to be 0.62 and % RSD for repeatability of sample application was found to be 0.75 which confirms that the method is precise. The %recovery was in the range 99.45-100.37 %, indicating the method accuracy (Table 3). The LOD and LOQ were found to be 5.29ng/spot and 16.04 ng/spot respectively. The specificity of the method was ascertained by comparing spectra acquired at the start (S), apex (M), and end (E) of the band, r²(s,m) = 0.9997 and r²(m,e) = 0.9996. Good correlation (r² = 0.9996) was obtained between spectra acquired from DEM standard, forced degraded sample and formulation (Figure 9). The summary of the validation parameters is given in Table 4.

Analysis of Formulation

The drug content of capsule was found to be 98.061 % ± 1.66 for DEM of label claim, which indicated that the method is suitable for routine analysis of DEM in its formulation.

Analysis of Forced Degradation Studies

Forced degradation studies of DEM were carried out under various stress conditions and the chromatograms of acidic, alkaline, oxidative, neutral, photolytic and dry heat conditions are shown in Figures 3, 4, 5, 6, 7 and 8 respectively. The degradation of drug

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results of DEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range</td>
<td>50-250 ng/spot</td>
</tr>
<tr>
<td>Correlation Co-efficient (R²)</td>
<td>0.9955</td>
</tr>
<tr>
<td>Precision (intraday)</td>
<td>0.91-1.5 %RSD</td>
</tr>
<tr>
<td>Precision (interday)</td>
<td>1.21-1.7 %RSD</td>
</tr>
<tr>
<td>% Recovery</td>
<td>99.45-100.37%</td>
</tr>
<tr>
<td>Limit of Detection (LOD)</td>
<td>5.29 ng/spot</td>
</tr>
<tr>
<td>Limit of Quantification (LOQ)</td>
<td>16.04 ng/spot</td>
</tr>
<tr>
<td>Specificity</td>
<td>Specific</td>
</tr>
</tbody>
</table>

Table 4. Summary of validation parameters

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>% Drug degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N HCl at RT for ½ hr</td>
<td>32%</td>
</tr>
<tr>
<td>0.01N NaOH at RT for 5 min</td>
<td>80%</td>
</tr>
<tr>
<td>1% H₂O₂ at RT for 1 hr</td>
<td>33%</td>
</tr>
<tr>
<td>Water at RT for 8 hr</td>
<td>12%</td>
</tr>
<tr>
<td>Direct sunlight for 8 hr</td>
<td>10%</td>
</tr>
<tr>
<td>Dry heat at 110°C for 8 hr</td>
<td>10%</td>
</tr>
</tbody>
</table>

Table 5. Data for Degradation study
occurred extensively in acid-base hydrolysis and oxidative degradation whereas mild degradation occurred in neutral, thermal and dry heat condition (Table 5).
Figure 6. Degradation of DEM in water

Figure 7. Degradation of DEM in sunlight (photolytic)

Figure 8. Degradation of DEM in oven (dry heat)
CONCLUSIONS

High performance thin layer chromatography method has been developed to determine DEM in its pharmaceutical dosage form. The developed method was able to estimate DEM in presence of degradation products formed under different stress conditions. The % RSD of all precision study was found to be less than 2% and results of % recovery study was found to be in range of 98-102%. The absorbance reflectance spectra of standard, forced degraded sample and formulation were shown good correlation. The developed method was able to quantify the drug content accurately upto nanogram level. Hence the developed method is Stability indicating, precise, accurate, specific and sensitive for the estimation of DEM in its pharmaceutical dosage forms. The proposed method was applied for assay of DEM in marketed formulations and the assay results were found to be in good agreement with label claim. Hence the developed method can be applied for degradation kinetic study, dissolution study and quality control.

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